DETECTION AND PREVALENCE OF *BIBERSTEINIA TREHALOSI* IN FEEDLOT CATTLE

A Thesis

Presented to the

Faculty of the College of Graduate Studies and Research

Angelo State University

In Partial Fulfillment of the

Requirements for the Degree

MASTER OF SCIENCE

by

REAGAN ALI MITCHELL

May 2018

Major: Animal Science

DETECTION AND PREVALENCE OF *BIBERSTEINIA TREHALOSI* IN FEEDLOT CATTLE

by

REAGAN ALI MITCHELL

APPROVED:

Dr. Micheal W. Salisbury

Dr. Loree A. Branham

Dr. Chase A. Runyan

Dr. Allison Dushane

May 2, 2018

APPROVED:

Dr. Susan E. Keith
Dean, College of Graduate Studies and Research

ACKNOWLEDGEMENTS

Thank you to Texas Vet Lab, Inc. located in San Angelo, Texas, for funding the research project, and to the staff of the laboratory for their assistance with sample handling and testing. Additionally, thank you to the feed yard management for allowing samples to be taken, and to the feedlot personnel that assisted with the handling of the cattle and the sample collection process. I would also like to thank the San Antonio Livestock Exposition for providing financial assistance, making it possible to continue my educational endeavors. Lastly, I would like to thank my advisor, Dr. Salisbury, as well as, the rest of the faculty of the Department of Agriculture at Angelo State University.

ABSTRACT

The objective of this study was to determine the prevalence of *Bibersteinia trehalosi* (*B. trehalosi*) in feedlot cattle, as well as develop an improved method of detection via quantitative Polymerase Chain Reaction (qPCR). Nasopharyngeal swab samples were taken from cattle, located in five different Kansas feedlots, providing 188 samples. The samples were tested for *B. trehalosi*, *Mannheimia haemolytica, Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* by conventional culture methods. DNA was extracted from the original swab sample transport media. After extraction the samples were run on qPCR using an assay that was developed as part of this study. It was determined that the assay will need further optimization to enhance the sensitivity and specificity to *B. trehalosi*. Overall one *B. trehalosi* was detected, indicating a low prevalence among the samples analyzed.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

ABSTRACT iv

TABLE OF CONTENTS v

LIST OF TABLES vi

INTRODUCTION 1

LITERATURE REVIEW 2

MATERIALS AND METHODS 6

RESULTS 15

DISCUSSION 30

LITERATURE CITED 35

APPENDIX I 39

APPENDIX II 40

LIST OF TABLES

Table 1: Frequency and Percentage of Sample Types at Each Feed Yard (*n*=188) 7

Table 2: Frequency and Percentage of Organisms Found within Each Feed Yard (*n*=186) 16

Table 3: Frequency and Percentage of Organisms Found within Each Sample Type (*n*=186)...17

Table 4: Frequency and Percentage of *Mycoplasma* Presence in Samples Testing Positive for Additional Organisms (*n*=186) 18

Table 5: Frequency and Percentage of *Mycoplasma* Presence in Samples Testing Positive for Additional Screened Organisms from each Feed Yard (*n*=186).....20

Table 6: Frequency and Percentage of *Mycoplasma* Presence in Samples Testing Positive for Additional Screened Organisms from each Sample Type (*n*=186) 21

Table 7: Frequency and Percentage of *Mycoplasma* Positive Samples Found in Each Sample Type at Each Feed Yard (*n*=186).....23

Table 8: Frequency and Percentage of Organisms Found in Each Sample Type at Each Feed Yard (*n*=186).....25

Table 9: Results of All Tests Done on Samples with Possibility of Being *Bibersteinia trehalosi* 29

INTRODUCTION

The animal health sector is a major part of the agriculture industry. Biological and Pharmaceutical companies are constantly working to stay on the cutting edge of treatments for disease and implementing preventative measures to maintain herd health. As time goes on, an increasing number of pathogens are recognized as being a cause for an animal becoming ill. With progressing research, and a development of knowledge for disease-causing microorganisms, comes the need to determine what infectious agents should be of primary concern for producers, veterinarians, and biological/pharmaceutical manufacturers.

Prevalence data is an important factor in determining what pathogens should be of concern. The more often a specific microorganism is recognized and confirmed as the cause of a disease the greater the need for a vaccine. Prevalence studies are also essential in gaining more knowledge on an emerging infectious agent that may have limited literature pertaining to it. Animal health companies take prevalence data, and other studies associated with the pathogen in question, into consideration when deciding to develop a new product.

Advancements in diagnostic and molecular biology techniques are necessary for animal health companies to stay on the forefront of medicine. In order to obtain data relating to a pathogen, multiple diagnostic tools can be used, such as polymerase chain reaction (PCR), quantitative PCR (qPCR), genetic sequencing, etc. These techniques vary in the amount of time and expense it takes to garner results. Currently, qPCR is generally recognized as a quicker and more cost efficient technique. Therefore, development of qPCR assays against new or emerging microorganisms is paramount.

Journal of Animal Science

LITERATURE REVIEW

Bovine respiratory disease complex (BRDC) is the leading cause of morbidity and mortality among feedlot cattle in the United States and Canada, as well as exported cattle in Australia. This results in economic losses due to the cost of treatments, and a reduction in the feed efficiency and overall productivity of the cattle. Some clinical signs of BRDC include: fever, coughing, loss of appetite, depression, shallow breathing, and nasal and eye discharge. There are multiple factors that can lead to the onset of BRDC including; environmental stressors, travel related stress, and comingling of infected cattle (Holman et al., 2015; Moore et al., 2015; Anton et al., 2016). Several pathogens are considered to be involved in the complex, allowing for the manifestation of a respiratory infection. These pathogens include a number of both viruses and bacteria.

The viruses associated include: *Bovine herpesvirus* (BHV), *Bovine respiratory syncytial virus* (BRSV), *Bovine viral diarrhea virus* (BVDV), *Bovine parainfluenza virus 3* (PI-3), bovine coronavirus (BoCV), bovine rhinoviruses, and bovine adenoviruses. Of these BHV, BRSV, and PI-3 are the most common respiratory pathogens. Additionally, BVDV is a significant predisposing pathogen (Moore et al., 2015). Studies have been published worldwide looking into prevalence data of BRSV. These studies have found that BRSV is present in herds all across the globe and can also prompt secondary infections brought on by bacteria (Sacco et al., 2014).

The bacteria most commonly connected to BRDC consist of: *Mannheimia haemolytica* (*M. haemolytica*), *Histophilus somni* (*H. somni*), *Pasteurella multocida* (*P. multocida*), *Bibersteinia trehalosi* (*B. trehalosi*), *Mycoplasma bovis* (*M. bovis*), and *Arcanobacterium pyogenes* (Confer, 2009; Holman et al., 2015; Moore et al., 2015). All of

these bacteria are considered to be opportunistic pathogens, taking advantage of a weakened immune system. They are housed within the nasopharynx of the cattle as commensals, and do not cause illness until the immune system is compromised by either a viral infection or stress, thus allowing for the bacteria to enter the lungs (Confer, 2009; Holman et al., 2015; Anton et al., 2016). The most recent bacteria added to the list of BRDC pathogens is *B. trehalosi* (Confer, 2009).

Bibersteinia trehalosi is a gram negative, rod-shaped, nonmotile bacterium that is commonly found in bighorn sheep (*Ovis canadensis*) and ruminants worldwide (Blackall et al., 2007; Villard et al., 2008; Kugadas et al., 2016). On a blood agar plate *B. trehalosi* colonies appear to be greyish or yellowish in color, round in configuration, and semi-transparent at the periphery with beta-hemolysis present (Blackall et al. 2007). *Bibersteinia trehalosi* was formerly classified as *Pasteurella haemolytica* biotype T and *M. haemolytica* was classified as *Pasteurella haemolytica* biotype A. The difference separating the two being that *B. trehalosi* ferments trehalose and *M. haemolytica* utilizes arabinose (Blackall et al., 2007, Villard et al., 2008). This bacterium is a known cause of pneumonia as well as septicemia in bighorn sheep (Besser et al., 2012; Dassanayake et al., 2013). Additionally, it is the most common bacteria found in the tonsils of clinically normal American bison (Bowersock et al., 2014). *Mannheimia haemolytica* and *B. trehalosi* are the two most common pathogens isolated from pneumonia cases in sheep, with *B. trehalosi* being isolated more frequently than the former (Dassanayake et al., 2013; Drew et al., 2013; Kugdas et al., 2016). It is believed that capsular serotypes T3, T3, and T10 are responsible for causing disease when leukotoxin is secreted (Anton et al., 2016, Villard et al., 2012).

Recently there have been cases of *B. trehalosi* presence in cattle with respiratory disease, as well as causing hemorrhages in the epicardium, and a case of Subcutaneous Botryomycosis (Spagnoli et al., 2012; Harhay et al., 2014; British Veterinary Association Veterinary Record, 2015). Signs of an infection of *B. trehalosi* range from pneumonia in cattle to sudden death with lungs often having exudative fibrinous pneumonia, bronchiolitis, and alveolitis. It has also been implied in trade journal articles that the bacterium is on the rise (Newport 2017). Unpublished diagnostic laboratory data from Texas Vet Lab Inc. (TVL) in San Angelo, Texas suggests that *B. trehalosi* can be found in the lungs of cattle. This is also concurrent with cattle of the United Kingdom (Bowersock et al. 2014). In February of 2015, the Animal and Plant Health Agency (APHA), located in the United Kingdom, published an October of 2014 disease surveillance report. In this report, there were six out of a group of 20 market-bought calves that died due to an infection of *B. trehalosi*.

One objective of this study was to determine the prevalence of *B. trehalosi* in nasopharyngeal samples of feedlot cattle. This was done in order to be an aid in determining whether or not the bacteria can be considered a primary pathogen in relation to the Bovine Respiratory Disease Complex.

Prevalence data has been reported for *M. haemolytica, P. multocida, H. somni,* and *M. bovis* but not for *B. trehalosi* (Confer, 2009; Holman et al., 2015). In order for there to be a proper representation of *B. trehalosi* prevalence data, a time efficient form of detection is needed. This is the second objective of the study; to develop and validate a one-step quantitative polymerase chain reaction (qPCR) assay specific to *B. trehalosi*. The current method of detection, developed by Dassanayake et al. (2009), is a multiplex assay that was

developed using genes from both *B. trehalosi* and *M. haemolytica*, creating primers for each species based on the regions of least similarity. The primers are used with PCR and then samples are examined using gel electrophoresis. Deciphering whether the sample was *M. haemolytica* or *B. trehalosi* is based on where bands form in the gel. This current method is a time consuming process, and a qPCR assay has the potential to significantly decrease the time it takes to identify a *B. trehalosi* positive sample in diagnostic laboratories.

MATERIALS AND METHODS

Sampling for this study was conducted under veterinary supervision, and no experimental research was conducted directly on the cattle themselves. It was performed as a part of standard commercial husbandry practices under standard conditions; therefore, the IACUC committee approval was waived by the Angelo State University IACUC committee chair.

Sample Collection

Nasopharyngeal swab samples were collected by feedlot personnel from 188 feedlot cattle of varying health and age, in order to gather data for analysis. Five different feedlots in Kansas were visited, and the cattle sampled were broken down into three different types: fresh cattle, new pull, and repeat. A fourth type of 'N/A' was assigned to samples that were mailed into the lab with no information on the cattle type. 'Fresh Cattle' are those considered to be clinically healthy and continuing feed yard vaccination protocol, or those who are new to the feed yard and are beginning the protocol. 'New Pull' cattle were those that were brought to the hospital pen for the first treatment based on usual clinical signs of Bovine Respiratory Disease Complex (BRDC) by trained feedlot personnel. 'Repeat' cattle are those that have been brought to the hospital pen at least once before for BRDC treatment, returned to their original pen, then brought back to the hospital pen due to visual BRDC symptoms again. Table 1 displays the distribution of samples taken from each feedlot visited, Location 2 had the greatest proportion of samples taken at 84, followed by Location 1, Location 3, Location 4, and Location 5. Table 1 also shows the percentage that each type of sample made up for each individual feed yard, as well as, the number and percentage each feed yard and sample type made up of the entire sample set. Out of the four different types

Table 1: Frequency and Percentage of Sample Types at Each Feed Yard (*n*=188)

	Location 1			Location 2			Location 3			Location 4			Location 5			Total	
Type[1]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	F[5]	P[6]
Fresh Cattle	0	0.00	0.00	23	27.38	12.23	11	31.43	5.85	17	89.47	9.04	0	0.00	0.00	51	27.13
New Pull	4	9.52	2.13	12	14.29	6.38	6	17.14	3.19	2	10.53	1.06	4	50.00	2.13	28	14.89
Repeat	38	90.48	20.21	49	58.33	26.06	4	11.43	2.13	0	0.00	0.00	4	50.00	2.13	95	50.53
N/A	0	0.00	0.00	0	0.00	0.00	14	40.00	7.45	0	0.00	0.00	0	0.00	0.00	14	7.45
Total	42	100.00	22.34	84	100.00	44.68	35	100.00	18.62	19	100.00	10.11	8	100.00	4.26	188	100.00

[1]Fresh Cattle = clinically healthy and continuing feed yard vaccination protocol, or are new to the feed yard, New Pull = cattle that have been brought to the hospital pen for the first treatment based on clinical signs of BRDC, Repeat = brought to the hospital pen at least once before for BRDC treatment, N/A = samples mailed into the diagnostic lab with no reference to the state of the cattle they came from

[2]A = Frequency of the sample type from each specific feed yard with the row titled total being the total number of samples from each feed yard

[3]B = Percentage of the samples from the specific feed yard that were the corresponding sample type

[4]C = Percentage of the entire sample size with the row titled total being the percentage each feed yard made up of the entire sample size

[5]F = Total frequency of the sample type

[6]P = Total percentage each sample type made up of the entire sample size

of samples the greatest amount (50.53%) were classified as repeats. The swabs used to collect the samples were BBL CultureSwab Collection and Transport System swabs from Becton Dickinson (Sparks, MD). Following sample collection, swabs were kept on ice and shipped overnight to the diagnostic lab located in San Angelo, TX. They were then processed by removing the transport media from the original collection tube and placing it in a 2ml snap cap micro centrifuge tube (FisherScientific). One milliliter of 10% glycerol was then added to the original collection tube. This step was done in order to preserve the initial swab sample for future use on culture plates. The micro centrifuge tubes were then stored at -80°C until further examination. The original swabs were also stored at -80°C. The samples of the transport liquid in the micro centrifuge tubes underwent gram negative DNA extraction using the Wizard Genomic DNA Purification Kit by Promega (Product code number A1120). The Wizard Genomic DNA Purification Kit Quick Protocol FB022 was used for the extraction process. Following DNA extraction, samples were analyzed on quantitative PCR (qPCR) using the Applied Biosystems 7500 Fast Real-Time PCR (Foster City, CA) machine.

PCR Method Development

The first step of designing the custom qPCR assay was conducted using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website. The *O*-sialoglycoprotein endopeptidase (*gcp*) and manganese-dependent superoxide dismutase (*sodA*) portions were the initial starting places of the *Bibersteinia trehalosi* (*B. trehalosi*) and *Mannheimia haemolytica* (*M. heamolytica*) genome comparison. When that was found to be inadequate for this study, the process moved on to one of the four full genomes of *B. trehalosi* in Genbank, (Genbank accession number

NZ_CP006954.1) sequenced by Harhay et al. (2014) and Kugadas et al. (2016). The genome was selected and searched against the *Mannheimia* taxon using the nucleotide to nucleotide alignment. It was then compared to a full genome of *M. haemolytica* (Genbank accession number CP006957) to narrow down to the area of least similarity. By visually analyzing the alignment of the two genomes, the sections of DNA that looked to be least similar were noted by the base pair (bp) number location starting with section bp400,021 to bp500,100. That specific section of sequence was then entered into the BLAST tool and was again blasted against the *Mannheimia* taxon, resulting in identification of two smaller segments of DNA sequence that were visually dissimilar: bp420,001 through bp450,060 and bp460,021 through bp470,100. The process was continued in this format until three segments of approximately 100 to 500 base pairs were found: bp432,809 through bp432,978, bp460,021 through bp460,469, and bp466,546 through bp466,643. These portions of least similarity were then found within the full genome of *B. trehalosi* using the 'show sequence' function of the genbank reference page. The bp numbers were highlighted and compared to the gene locations in order to identify what gene is coded for in that segment; the included the PTS Trehalose transporter subunit IIBC, SH1A/HecA/FhaA exoprotein, and SMI1/KNR4 family protein respectively.

The sections of the DNA sequence were then entered into the Primer Express software to produce a primer/probe set compatible with the qPCR machine. Once a primer/probe set had been selected the entire amplicon region was entered into the alignment tool. This was used to identify any other potential organisms the assay could amplify, resulting in a false positive for *B. trehalosi*. After analyzing the possible primer sets, the portion selected for use came from the PTS Trehalose transporter subunit IIBC. The primer

set included a forward primer sequence of 5’ TTTTCCTTGCTCACGCCATTA 3’, a probe sequence of 5’ CGGTCCAATTGGG 3’, and a reverse primer sequence of 5’AACCCCATTGCCAATTTCG 3’. Total amplicon length being 56 base pairs long.

The forward and reverse primers were first ordered from Integrated DNA Technologies custom oligo order in the 100nmole scale. In order to optimize the primer set protocol, they were tested on regular Polymerase Chain Reaction (PCR). The PCR master mix consisted of 5µl of 10X Std Taq Rxn Buffer (New England BioLabs, product number B9014S) per reaction, 1µl of 10mM dNTP (Applied Biosystems, product number N8080260) per reaction, 1µl of 10µM forward primer per reaction, 1µl of 10µM reverse primer per reaction, and 0.25µl of Taq DNA Polymerase (New England BioLabs, product number M0273S) per reaction. Magnesium chloride was added to the master mix at a rate of 1.5µl per reaction when needed. Each well used in the PCR contained 8.25µl of the master mix, 1-2µl of extracted sample depending on DNA concentrations (~100ng of DNA per reaction), and the appropriate amount of water to bring the final volume of the well to 50µl. The final PCR parameters for the assay were set at a holding stage of 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds, 66°C annealing temperature for 1 minute and extension at 72°C for 1 minute, followed by 72°C for 5 minutes and finishing at 4°C for infinity. Samples of *B. trehalosi*, *M. haemolytica* A1, *M. haemolytica* A2, *M. haemolytica* A6, *Pasteurella multocida* (*P. multocida*), and *Histophilus somni* (*H. somni*) were used in the PCR run to determine if the primer set was capable of amplifying an organism other than *B. trehalosi*. Following amplification, the test samples were analyzed on a 1.5% agarose gel using Tris-Acetate-EDTA (TAE) buffer.

Once the run parameters were optimized, and preliminary confirmation that the

primers would only amplify *B. trehalosi* was established, the assay was ordered through ThermoFisher with the probe included to be used with the qPCR machine as a simplex assay. The master mix for the simplex reactions were made by using 5µl of 1-Step Master Mix per reaction, 1µl of the 20X assay per reaction, and 12µl of RNAse-Free water per reaction. The plates were loaded with 18µl of the master mix and 2µl of extracted DNA. The assay was then optimized on the 7500 fast machine starting with the run parameters of the PCR and going up one degree on the annealing temperature until *B. trehalosi* was the only organism of the six tested with a positive threshold cycle score (C_t). If the C_t scores were 30 or less at a threshold of 0.5 it was considered a positive result. The final run parameters were a holding stage of 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds 68°C annealing temperature for 1 minute and extension at 72°C for 1 minute.

Sensitivity of the assay was tested by growing a known *B. trehalosi* isolate culture from the Texas Vet Lab, Inc. (TVL) diagnostic lab inventory in Tryptic Soy Broth with Tryptose (TSTB; see Appendix I). The bacteria isolate was first streaked out onto a Trypticase™ Soy Agar with 5% Sheep Blood (TSA II) plate from FisherScientific. The plate was allowed to incubate at 37°C in a 5% CO_2 incubator for approximately 15 hours. After, the isolate was aseptically transferred to the seed tube by utilizing a sterile loop. The tube was then incubated at 37°C in a 5% CO_2 incubator for approximately 15hours. Eight 1:10 serial dilutions were performed using Phosphate-Buffered saline (PBS). All samples were extracted using the same Promega kit as previously mentioned and run on qPCR. A colony count was taken on the initial sample from the TSTB tube.

Traditional Culture Methods

As the extracted samples were run on PCR, the initial swab samples that were frozen

in the 10% glycerol were placed on Trypticase Soy Agar with 5% Sheep Blood (TSA II) plates and incubated for approximately 15 hours at 37°C in a 5% CO_2 incubator. After incubation, the plates were visually examined by trained lab personnel for bacterial growth; colonies were streaked for isolation on a new plate if needed, and biochemical tests of indole, oxidase, and catalase reaction were tested. When the colony appeared gray in color, semi-transparent at the periphery, and was beta-hemolytic, as well as possessed the desired biochemical results, it was considered to be a potential *B. trehalosi*. Colony morphology for *M. haemolytica* is similar to that of *B. trehalosi* with smooth grayish colonies and light hemolysis. *P. multocida* colonies appear as a grayish color as well but are mucoidal and no presence of hemolysis. *H. somni* colonies are small with yellow pigment and no hemolysis.

Approximately 100µl of the glycerol put in the original swab sample was transferred onto a heart infusion agar base Mycoplasma agar plate (see Appendix II), by using a transfer pipette covering three-fourths of the plate. The plates were then incubated at 37°C for 48 hours in a 5% CO_2 incubator to determine if *Mycoplasma bovis* was present in the sample. Typical *Mycoplasma* colonies are very small, circular in form, appear to have a gray peripheral zone on the surface of the agar, and a yellowish centralized zone embedded in the agar, commonly referred to as a "fried egg". A standard light microscope must be used to examine the plates due to the small size of the colonies.

Confirmation Testing

Primary data collected for the PCR development included the C_t score, which was used to determine if the sample was positive for *B. trehalosi*. This was then further confirmed by assessing the bacterial growth of the blood plate by using biochemical test reactions looking for a result of indole negative, oxidase positive, and catalase negative.

The previous master mix and PCR protocol mentioned was followed with modifications by using *M. haemolytica* primers as well as modifying the annealing temperature and cycles for *M. haemolytica* detection. The potential *B. trehalosi* samples were visualized on gel electrophoresis for further confirmation by determining if the samples were *M. haemolytica* positive or negative. The samples were grown on a plate and then in TSTB as previously mentioned prior to PCR. If no bands were formed in the gel, the possibility of the sample being a *M. haemolytica* was ruled out.

The samples that were considered positive for *B. trehalosi* through culture methods and PCR were serotyped using typing sera from TVL inventory provided by the United States Department of Agriculture. A loopful of bacteria was used to make a suspension by combining with half a milliliter of sterile water in a micro centrifuge tube and mixing by vortex. A plate agglutination slide was used by placing approximately 20µl of sterile water and the typing serums each in their own respective circle. Seventy to 100µl of suspension was gently added and mixed to each serum being tested as well as the negative control circle containing water. The plate was then gently rocked in a circular motion and looked at over light in a dark room to determine if clumping was present. Positive agglutination in the various test circles indicated serotype confirmation.

Before all PCR runs, the extracted DNA had to be quantified using a Quibit fluorometer. Manufacturer protocol for the Qubit™ dsDNA BR Assay Kit (ThermoFisher Scientific) was followed for this process.

After all samples had been tested for the initial data collection of the study, a different DNA extraction option was tested to see what the effect on the sensitivity of the qPCR assay would be. A positive sample of *B. trehalosi* from TVL inventory was grown on a plate and

in TSTB following the procedure previously mentioned. Eight 1:10 serial dilutions were made, and a colony count was conducted. The samples were then extracted using both the Promega kit as well as the DNeasy Blood & Tissue Kit (Cat No./ID: 69504) by Qiagen (Germantown, MD). The manufacturer protocol for the Qiagen kit was followed with modifications in the first two steps. Modifications included spinning down 1ml of bacterial liquid sample at 5660 x *g* for 10 minutes. The supernatant was removed and 180µl of Buffer Animal Tissue Lysis and 20µl of proteinase K were added and vortexed. The samples were then incubated in a water bath of 56°C for approximately 1 to 2 hours with intermittent mixing by the vortex approximately every 10 to 15 minutes. The two sets of extracted DNA were then tested on qPCR and compared.

Statistical Analysis

Collected data was analyzed using the frequency procedure of SAS (PROC FREQ) (SAS 9.1.3) in order to compile descriptive statistics. As part of this, a Chi-Square test was run in order to determine statistical differences in *Mycoplasma bovis* presence among feed yards.

RESULTS

Organism Prevalence

Of the 188 swab samples taken, 186 were put onto Trypticase Soy Agar with 5% Sheep Blood (TSA II) plates and heart infusion agar base Mycoplasma agar plates. Table 2 provides the frequency and percentage of samples that were found to have the presence of *Mycoplasma*, as well as the samples that were found to have an additional pathogen, for each feed yard. The table also indicates the percentage of each organism result as part of the entire set of samples. Table 3 presents similar data for each sample type class.

Fifty percent of the samples tested (93 of 186) were positive for *Mycoplasma bovis*; 20.43% of which came from samples classified as Repeat. Additionally, 22.04% of the positive samples came from Location 2. There was no statistical difference among positive *Mycoplasma* tested samples between feed yards ($p = 0.75$). The TSA II culture plates were examined for four potential pathogens: *B. trehalosi, H. somni, M. haemolytica,* and *P. multocida*. *Mannheimia heamolytica* was found at the greatest frequency of all the pathogens with a total of 30 presumptive positive *M. haemolytica* plates (16.13%). This was followed by *H. somni*, *P. multocida*, and *B. trehalosi* at 5.38%, 4.30%, and 0.54% presumptive positive plates respectively. However, 73.66% of the total samples showed no visual presence of the pathogens on the TSA II plate. These results can be examined along with the percentages of each microorganism in Table 2 and Table 3.

Table 4 is a representation of the amount of *Mycoplasma* presence within each organism result. The one *B. trehalosi* sample was negative for *Mycoplasma*. *Mannheimia haemolytica* and *H. somni* were found more often in *Mycoplasma* negative samples than *Mycoplasma* positive samples. Seven of the eight *P. multocida* samples were positive for

Table 2: Frequency and Percentage of Organisms Found within Each Feed Yard (*n*=186)

	Feed Yard																
	Location 1			Location 2			Location 3			Location 4			Location 5			Total	
Organism	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	F[4]	P[5]
TSA II Plate[6]																	
Bt	0	0.00	0.00	1	1.22	0.54	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	1	0.54
Hs	3	7.14	1.61	4	4.88	2.15	3	8.57	1.61	0	0.00	0.00	0	0.00	0.00	10	5.38
Mh	8	19.05	4.30	13	15.85	6.99	3	8.57	1.61	4	21.05	2.15	2	25.00	1.08	30	16.13
Pm	0	0.00	0.00	2	2.44	1.08	5	14.29	2.69	0	0.00	0.00	1	12.50	0.54	8	4.30
Neg.	31	73.81	16.67	62	75.61	33.33	24	68.57	12.90	15	78.95	8.06	5	62.50	2.69	137	73.66
Total	42	100.00	22.58	82	100.00	44.09	35	100.00	18.82	19	100.00	10.22	8	100.00	4.30	186	100.00
Myco[7]																	
Pos.	17	40.48	9.14	41	50.00	22.04	20	57.14	10.75	10	52.63	5.38	5	62.50	2.69	93	50.00
Neg.	22	52.38	11.83	35	42.68	18.82	15	42.86	8.06	9	47.37	4.84	3	37.50	1.61	84	45.16
Con.	3	7.14	1.61	6	7.32	3.23	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	9	4.84
Total	42	100.00	22.58	82	100.00	44.09	35	100.00	18.82	19	100.00	10.22	8	100.00	4.30	186	100.00

[1]A = Frequency of samples from each feed yard that possessed the corresponding organism result with the row titled total equaling the total number of samples from that feed yard
[2]B = Percentage of samples from each feed yard that possessed the corresponding organism result
[3]C = Percentage of the total number of samples put on each specific type of culture plate with the row titled total equaling the percentage each feed yard made up of the entire sample size tested
[4]F = Total frequency of each organism result
[5]P = Total percentage each organism result made up for the entire set of samples tested on the specific type of agar plate
[6]Trypticase Soy Agar with 5% Sheep Blood; *Bt = Bibersteinia trehalosi, Hs = Histophilus somni, Mh = Mannheimia haemolytica, Pm = Pasteurella multocida,* Neg. = Negative
[7]*Mycoplasma bovis*; Pos. = Positive, Neg. = Negative, Con. = Contaminated

Table 3: Frequency and Percentage of Organisms Found within Each Sample Type (*n*=186)

	Sample Type[1]													
	Fresh Cattle			New Pull			Repeat			N/A			Total	
Organism	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	F[5]	P[6]
TSA II Plate[7]														
B. trehalosi	0	0.00	0.00	0	0.00	0.00	1	1.08	0.54	0	0.00	0.00	1	0.54
H. somni	3	5.88	1.61	3	10.71	1.61	3	3.23	1.61	1	7.14	0.54	10	5.38
M. haemolytica	5	9.80	2.69	5	17.86	2.69	19	20.43	10.22	1	7.14	0.54	30	16.13
P. multocida	2	3.92	1.05	1	3.57	0.54	1	1.08	0.54	4	28.57	2.15	8	4.30
Negative	41	80.39	22.04	19	67.86	10.22	69	74.19	37.10	8	57.14	4.30	137	73.66
Total	51	100.00	27.42	28	100.00	15.06	93	100.00	50.01	14	100.00	7.53	186	100.00
Mycoplasma bovis														
Positive	21	41.18	11.29	22	78.57	11.83	38	40.86	20.43	12	85.71	6.45	93	50.00
Negative	29	56.86	15.59	6	21.43	3.23	47	50.54	25.27	2	14.29	1.08	84	45.16
Contaminated	1	1.96	0.54	0	0.00	0.00	8	8.60	4.30	0	0.00	0.00	9	4.84
Total	51	100.00	27.42	28	100.00	15.06	93	100.00	50.01	14	100.00	7.53	186	100.00

[1]Fresh Cattle = clinically healthy and continuing feed yard vaccination protocol, or are new to the feed yard, New Pull = cattle that have been brought to the hospital pen for the first treatment based on clinical signs of BRDC, Repeat = brought to the hospital pen at least once before for BRDC treatment, N/A = samples mailed into the diagnostic lab with no reference to the state of the cattle they came from

[2]A = Frequency of samples from each sample type that possessed the corresponding organism result, with the row titled total equaling the total number of samples from that sample type

[3]B = Percentage of samples from each sample type that possessed the corresponding organism result

[4]C = Percentage of the total number of samples put on each specific type of culture plate with the row titled total equaling the percentage each sample type made up of the entire sample size tested

[5]F = Total frequency of each organism result

[6]P = Total percentage each organism result made up for the entire set of samples tested on the specific type of agar plate

[7]Trypticase Soy Agar with 5% Sheep Blood; *B. trehalosi = Bibersteinia trehalosi*, *H. somni = Histophilus somni*, *M. haemolytica = Mannheimia haemolytica*, *P. multocida = Pasteurella multocida*

Table 4: Frequency and Percentage of *Mycoplasma* Presence in Samples Testing Positive for Additional Organisms (*n*=186)

	Myco Agar Plate[1]		
Organism	Frequency	Percentage of Organism[2]	Percentage of Entire[3]
Bibersteinia trehalosi	0	0.00	0.00
Histophilus somni	3	30.00	1.61
Mannheimia haemolytica	12	40.00	6.45
Pasteurella multocida	7	87.50	3.76
Negative[4]	71	51.82	38.17
Total	93		50.00

[1] Heart infusion agar supplemented with heart infusion broth, thallium acetate, and PP #3
[2]Percentage of *Mycoplasma* presence in the samples testing positive for the specific organism result
[3]Percentage of total Myco Agar Plates testing positive for *Mycoplasma*
[4]Negative for screened organisms

Mycoplasma. The majority of the *Mycoplasma* positive samples came from swabs that were identified as negative for the four pathogens of interest. Out of the 93 samples that tested positive for *Mycoplasma*, 71 were negative for the other screened organisms (38.17%). Nine out of the 186 samples were too contaminated to evaluate for *Mycoplasma* presence.

Table 5 and Table 6 examine the *Mycoplasma* results for each organism within each feed yard and sample type respectively. Out of the 42 samples taken at Location 1, 40.48% of the samples were positive for *Mycoplasma*, of which 41.94% were negative for additional potential pathogens. When comparing the percentages of positive *Mycoplasma* samples across the five feed lots, Location 1 had the lowest percentage of its samples test positive. Location 5 had the highest percentage of its total samples test positive for *Mycoplasma* at 62.50%, which were five out of the eight samples from that yard. Four of the five *Mycoplasma* positive samples were also found to be negative for the additional pathogens of interest. Location 3 was the next highest in the percentage of positive *Mycoplasma* samples followed by Location 4 and then Location 2. Location 2 had half of the 82 samples test positive for *Mycoplasma*. Most of the samples from Location 2 that were positive for *Mycoplasma* were not found to have one of the other four screened pathogens (33 out of the 41). There were 35 samples from Location 3 tested and 57.14% were positive for *Mycoplasma*. All five of the *P. multocida* samples from Location 3 were positive for *Mycoplasma*. Location 4 had 10 out of 19 samples test positive for *Mycoplasma*, which was 52.63% of the total for that yard. Within Location 4 there were four plates that were presumptive positive for *M. haemolytica*, two of them were positive for *Mycoplasma*.

Out of the four sample types, the N/A samples had the largest percentage of its samples test positive for *Mycoplasma* at 85.71% (12 out of 14 samples). New Pull samples

Table 5: Frequency and Percentage of *Mycoplasma* Presence in Samples Testing Positive for Additional Screened Organism from each Feed Yard (*n*=186)

	Organism																
	Bibersteinia trehalosi			*Histophilus somni*			*Mannheimia haemolytica*			*Pasteurella multocida*			Negative				
Feed Yard	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	A[1]	B[2]	C[3]	Total Frequency	Total Percent[4]
Location 1 (*n*=42)	0	0.00	0.00	1	33.33	2.38	3	37.50	7.14	0	0.00	0.00	13	41.94	30.95	17	40.48[a]
Location 2 (*n*=82)	0	0.00	0.00	2	50.00	2.44	5	38.46	6.10	1	50.00	1.22	33	53.23	40.24	41	50.00[a]
Location 3 (*n*=35)	0	0.00	0.00	0	0.00	0.00	2	66.67	5.71	5	100.00	14.29	13	54.17	37.14	20	57.14[a]
Location 4 (*n*=19)	0	0.00	0.00	0	0.00	0.00	2	50.00	10.53	0	0.00	0.00	8	53.33	42.11	10	52.63[a]
Location 5 (*n*=8)	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	1	100.00	12.50	4	80.00	50.00	5	62.50[a]

[1]A = Frequency of samples from each feed yard that were positive for specific screened organisms that were also positive for *Mycoplasma*
[2]B = Percentage of the samples from each feed yard positive for specific screened organisms that were also positive for *Mycoplasma*
[3]C = Percentage of the entire samples from each individual feed yard that were positive for *Mycoplasma*
[4] Total Percentage of samples from the corresponding feed yard that were positive for *Mycoplasma*
[a] Chi-Square test statistically similar among feed yards sampled in percentage of positive *Mycoplasma* tested samples, p = 0.75

Table 6: Frequency and Percentage of *Mycoplasma* Presence in Samples Testing Positive for Additional Screened Organisms from each Sample Type (*n*=186)

	Organism																
	Bibersteinia trehalosi			*Histophilus somni*			*Mannheimia haemolytica*			*Pasteurella multocida*			Negative				
Sample Type[1]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	Total Frequency	Total Percent[5]
Fresh Cattle (*n*=51)	0	0.00	0.00	0	0.00	0.00	1	20.00	1.96	1	50.00	1.96	19	46.34	37.25	21	41.18
New Pull (*n*=28)	0	0.00	0.00	2	66.67	7.14	3	60.00	10.71	1	100.00	3.57	16	84.21	57.14	22	78.57
Repeat (*n*=93)	0	0.00	0.00	1	33.33	1.08	7	36.84	7.53	1	100.00	1.08	29	42.03	31.18	38	40.86
N/A (*n*=14)	0	0.00	0.00	0	0.00	0.00	1	100.00	7.14	4	100.00	28.57	7	87.50	50.00	12	85.71

[1]Fresh Cattle = clinically healthy and continuing feed yard vaccination protocol, or are new to the feed yard, New Pull = cattle that have been brought to the hospital pen for the first treatment based on clinical signs of BRDC, Repeat = brought to the hospital pen at least once before for BRDC treatment, N/A = samples mailed into the diagnostic lab with no reference to the state of the cattle they came from

[2]A = Frequency of samples from each sample type that were positive for specific screened organisms that were also positive for *Mycoplasma*

[3]B = Percentage of the samples from each sample type positive for specific screened organisms that were also positive for *Mycoplasma*

[4]C = Percentage of the entire samples from each individual sample type that were positive for *Mycoplasma*

[5] Total Percentage of samples from the corresponding sample type that were positive for *Mycoplasma*

had the next highest percentage of positive *Mycoplasma* samples at 78.57% of the 28 samples in that type. Of the 28 New Pull samples tested, 57.14% were negative for potential pathogens and 10.71% were positive for *M. haemolytica*. Fresh Cattle swabs had 41.18% positive for *Mycoplasma*. Only two of the 21 positive Fresh Cattle samples were found to have an additional microorganism of interest, one *M. haemolytica* and one *P. multocida.* The Repeat sample type had the greatest number of samples tested at 93; only 38 were found to have *Mycoplasma* present (40.86%). There were 19 positive *M. haemolytica* samples in the repeat sample type (20.43%); seven of which were positive for *Mycoplasma* (36.84%).

The presence of *Mycoplasma* is further evaluated in Table 7. The samples are broken down by both sample type and feed yard. Out of all the fresh cattle samples that were positive for *Mycoplasma* presence, 11 of them were taken at Location 2, which was 52.38% of samples for fresh cattle. Location 4 had 9 positive fresh cattle samples equating to 42.86%. The last 4.76% came from one sample taken at Location 3. There were a total of 22 new pull samples that were positive for *Mycoplasma*. Over 50% of these samples were from Location 2. The remaining 10 samples came from Location 3, Location 5, Location 1, and Location 4 in that order at four, three, two, and one positive new pull samples respectively. Of the 38 *Mycoplasma* positive repeat samples the majority came from Location 1 and Location 2 at 15 and 18 respectively. Location 1 made up 39.47%, Location 2 47.37%, and the remaining came from Location 3 and Location 5 at 7.89% and 5.26% respectively. All of the N/A samples were from Location 3. The 12 positive N/A samples (all from Location 3) made up 60.00% of the *Mycoplasma* positive samples for that location. New pull samples accounted for 20.00%, repeat 15.00%, and fresh cattle 5.00%. The majority of the positive samples from Location 1 were the 15 repeat samples at 88.24%. The remaining 11.76%

Table 7: Frequency and Percentage of *Mycoplasma* Positive Samples Found in Each Sample Type at Each Feed Yard (*n*=186)

	Sample Type[1]											
	Fresh Cattle			New Pull			Repeat			N/A		
Feed Yard	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]
Location 1	0	0.00	0.00	2	11.76	9.09	15	88.24	39.47	0	0.00	0.00
Location 2	11	26.83	52.38	12	29.27	54.55	18	43.90	47.37	0	0.00	0.00
Location 3	1	5.00	4.76	4	20.00	18.18	3	15.00	7.89	12	60.00	100.00
Location 4	9	90.00	42.86	1	10.00	4.55	0	0.00	0.00	0	0.00	0.00
Location 5	0	0.00	0.00	3	60.00	13.64	2	40.00	5.26	0	0.00	0.00

[1]Fresh Cattle = clinically healthy and continuing feed yard vaccination protocol, or are new to the feed yard, New Pull = cattle that have been brought to the hospital pen for the first treatment based on clinical signs of BRDC, Repeat = brought to the hospital pen at least once before for BRDC treatment, N/A = samples mailed into the diagnostic lab with no reference to the state of the cattle they came from

[2]A = Frequency of *Mycoplasma* positive samples for each of the different sample types within the samples gathered from each feed yard

[3]B = Percentage of the *Mycoplasma* positive samples for the entire feed yard that came from the corresponding sample type

[4]C = Percentage of the *Mycoplasma* positive samples from the entire sample type that came from the corresponding feed yard

came from new pull samples. Location 2 positives were distributed over three sample types; fresh cattle, new pull, and repeat at 26.83%, 29.27%, and 43.90% respectively. Almost all of the *Mycoplasma* positive samples from Location 4 came from fresh cattle at 90.00%. Location 5 was split between new pull and repeat samples, with repeat accounting for 40.00% and new pull accounting for 60.00%.

Table 8 is similar to Table 7 in that it is showing the same type of data except in relation to the different organisms found instead of *Mycoplasma* presence. As previously mentioned there was only one *B. trehalosi* found, so consequently 100.00% came from Location 2 and it was a repeat sample. Of the nine samples identified to have *H. somni* present, three were repeat samples from Location 1. These were the only three *H. somni* samples identified from those taken at Location 1; they were also the only three repeat samples to have *H. somni*. Location 2 had four samples with *H. somni* present, evenly distributed between fresh cattle and new pulls. These both made up 66.67% of the *H. somni* found in each respective sample type. Location 3 also had an even distribution of *H. somi* among fresh cattle and new pull sample types at one each. This made up the remaining 33.33% for the sample types.

There was a higher prevalence of *M. haemolytica* compared to the other screened organisms among the samples tested. All eight *M. haemolytica* positive samples from Location 1 were from repeat samples. Additionally, these eight made up 42.11% of *M. haemolytica* found among repeat samples. The *M. haemolytica* positives found within Location 2 samples were from fresh cattle, new pulls, and repeats with repeat having the highest number at 10, making up 76.92%. These 10 samples accounted for 52.63% of repeat samples positive for *M. haemolytica*. The fresh cattle *M. haemolytica* samples made up

Table 8: Frequency and Percentage of Organisms Found in Each Sample Type at Each Feed Yard (*n*=186)

	Organism														
	Bibersteinia trehalosi			*Histophlius somni*			*Mannheimia Haemolytica*			*Pasteurella multocida*			Negative		
Feed Yard/ Sample Type[1]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]	A[2]	B[3]	C[4]
Location 1															
Fresh Cattle	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
New Pull	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	4	12.90	21.05
Repeat	0	0.00	0.00	3	100.00	100.00	8	100.00	42.11	0	0.00	0.00	27	87.10	39.13
N/A	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
Location 2															
Fresh Cattle	0	0.00	0.00	2	50.00	66.67	2	15.38	40.00	1	50.00	50.00	18	29.03	43.90
New Pull	0	0.00	0.00	2	50.00	66.67	1	7.69	20.00	0	0.00	0.00	9	14.52	47.37
Repeat	1	100.00	100.00	0	0.00	0.00	10	76.92	52.63	1	50.00	100.00	35	56.45	50.72
N/A	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
Location 3															
Fresh Cattle	0	0.00	0.00	1	50.00	33.33	1	33.33	20.00	1	20.00	50.00	8	33.33	19.51
New Pull	0	0.00	0.00	1	50.00	33.33	1	33.33	20.00	0	0.00	0.00	4	16.67	21.05
Repeat	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	4	16.67	5.80
N/A	0	0.00	0.00	0	0.00	0.00	1	33.33	100.00	4	80.00	100.00	8	33.33	100.00
Location 4															
Fresh Cattle	0	0.00	0.00	0	0.00	0.00	2	50.00	40.00	0	0.00	0.00	15	100.00	36.59
New Pull	0	0.00	0.00	0	0.00	0.00	2	50.00	40.00	0	0.00	0.00	0	0.00	0.00
Repeat	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
N/A	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
Location 5															
Fresh Cattle	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
New Pull	0	0.00	0.00	0	0.00	0.00	1	50.00	20.00	1	100.00	100.00	2	40.00	10.53
Repeat	0	0.00	0.00	0	0.00	0.00	1	50.00	5.26	0	0.00	0.00	3	60.00	4.35
N/A	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00

[1]Fresh Cattle = clinically healthy and continuing feed yard vaccination protocol, or are new to the feed yard, New Pull = cattle that have been brought to the hospital pen for the first treatment based on clinical signs of BRDC, Repeat = brought to the hospital pen at least once before for BRDC treatment, N/A = samples mailed into the diagnostic lab with no reference to the state of the cattle they came from

[2]A = Frequency of the organisms identified for each of the different sample types within the samples gathered from each feed yard

[3]B = Percentage of the specific organism identified for the entire feed yard samples that came from the specific sample type

[4]C = Percentage of the specific organism identified from the entire sample type that came from the corresponding feed yard

15.38% of those from Location 2, and the remaining 7.69% was the one sample that was in the new pull category. Location 2 accounted for 40.00% of the entire *M. haemolytica* positive fresh cattle samples and 20.00% of the entire new pull samples positive for *M. haemolytica*. The *M. haemolytica* samples from Location 3 were evenly distributed among fresh cattle, new pull, and N/A each having one positive. This accounted for 20.00% of *M. haemolytica* positive samples for both fresh cattle and new pull sample types. Again there was an even distribution among sample types for both Location 4 and Location 5. Location 4 had two samples from fresh cattle and two from new pull samples that were positive for *M. haemolytica*. Both of these accounted for 40.00% of the positive samples for each respective type. Location 5 had one sample with *M. haemolytica* present in both new pull and repeat sample types. The one new pull made up 20.00% of all new pull *M. haemolytica* positive samples, and the one repeat accounted for 5.26% of all repeat *M. haemolytica* positive samples.

Pasteurella multocida was found in eight samples from three different feed yards and in all four sample types. At Location 2 there were two samples found to be positive, one fresh cattle sample and one repeat sample. This was the only repeat sample found to have *P. multocida,* and the Location 2 fresh cattle sample accounted for 50.00% of all *P. multocida* positive fresh cattle samples. Location 3 had five total *P. multocida* samples, four N/A sample types making up 80.00% of those from Location 3, and one fresh cattle sample type accounting for the remaining 20.00% for that feed yard and the remaining 50.00% for the sample type. Location 5 only had one sample show presence of *P. multocida*. This was a new pull sample, and was the only new pull sample to be positive for *P. multocida*. The distribution of feed yard and sample type for those samples found to be negative for the

primary pathogens is also shown in table eight.

qPCR Detection and Development

The extraction and qPCR detection process was performed on 186 of the 188 samples, as two of the original samples were lost during transport. Of the 186, two samples were able to report a threshold cycle (C_t) score all other samples had undetermined results at a threshold of 0.5. The C_t scores for the two positive samples were 34.43 and 30.65 respectively. All samples were evaluated again at the threshold automated by the qPCR run resulting in one other sample having a C_t score. This third sample's score was reported at 31.96. The three samples that garnered a C_t score were evaluated on the TSA II plates, and the colonies that were suspected to be *B. trehalosi* were isolated and grown on a separate plate and TSTB tubes, re-extracted and run on qPCR again. In total there were seven samples that were regrown and extracted a second time to be run on qPCR; these included the three with a C_t score from the original sample, and four that had a colony suspected to be *B. trehalosi* based on colony morphology and a negative catalase reaction.

The second run of PCR on these samples resulted in C_t values of 16.9 for one of the four original samples suspected to have a *B. trehalosi* colony and 27.55 for one of the original three samples that reported a C_t score, all others came back undetermined at a threshold of 0.5. When all seven of the retest samples were run on PCR and gel electrophoresis, no bands were formed, ruling out the possibility of the samples being *M. haemolytica*. All of the samples were also retested with the biochemical agents resulting in only one sample having the *B. trehalosi* results of indole negative, oxidase positive, and catalase negative. All other samples did not have biochemical results correlating with any of the four microorganisms of interest in this study. The one sample with a positive C_t at a

threshold of 0.5, the proper colony morphology, and proper biochemical test results, was one originally identified by suspected colony morphology. This sample had an agglutination test done in order to serotype, resulting in it being confirmed type T3 with a slight possibility of T10. Table 9 displays the results of all the tests run on the samples that were tested twice.

Sensitivity trials were run on the developed qPCR assay. The first test resulted in the original undiluted *B. trehalosi* extracted sample having a C_t value of 18.93 and the first log dilution 30.6, at a threshold of 0.5. The remaining seven dilutions came back undetermined. The second sensitivity trial had the following results at a threshold of 0.5: lightly inoculated undiluted sample of 19.25, light dilution one of 30.3, heavily inoculated undiluted sample of 16.05, and heavy dilution one of 26.83. Again, all other dilutions came back undetermined. The original colony count prior to dilution was 2.8×10^7.

The Qiagen extraction method was not run on qPCR because quantification of extracted DNA came back to be comparable to that of the Promega samples. DNA concentration levels of Qiagen extractions were at 8.84ng/µl of DNA for the lightly inoculated tube and 5.52ng/µl of DNA for the heavy inoculated tube compared to 2.35ng/µl and 4.68ng/µl for the Promega samples. However, the first dilution of the Qiagen extracted samples had a small amount of detectable DNA when quantified, whereas all of the dilutions for the Promega samples were too low to be detected.

Table 9: Results of All Tests Done on Samples with Possibility of Being *Bibersteinia trehalosi*

	Initial Tests[1]		Re-test[2]	Additional Tests[3]		
Sample	TSA II Plate[4] (suspected)	C_t[5]	C_t[5]	Biochemical Tests[6]	PCR/Gel[7]	Serotype
B957G	Negative	34.43	Undetermined	I: - O: - C: + (N/A)	No bands	
K 1147	(*B. trehalosi*)[8]	Undetermined	Undetermined	I: - O: - C: + (N/A)	No bands	
K 1991	*M. haemolytica*[9]	31.96[10]	27.55	I: - O: - C: + (N/A)	No bands	
K 4372	(*B. trehalosi*)[8]	Undetermined	16.9	I: - O: + C: - (*B. trehalosi*)[8]	No bands	T3 (possibly T10)
K 7029	Negative	30.65	Undetermined	I: - O: - C: - (N/A)	No bands	
K 2465	(*B. trehalosi*)[8]	Undetermined	Undetermined	I: - O: - C: + (N/A)	No bands	
PV 15	(*B. trehalosi*)[8]	Undetermined	Undetermined	I: - O: - C: - (N/A)	No bands	

[1] The tests conducted on the original swab and extracted sample
[2] The tests conducted on selected isolated colonies that were regrown after initial testing
[3] Additional tests conducted on the regrown samples in order to further confirm organism identification
[4] Trypticase Soy Agar with 5% Sheep Blood
[5] Threshold Cycle score at a threshold of 0.5
[6] I=indole, O=oxidase, C=catalase, - = Negative, + = Positive
[7] PCR assay for *Mannheimia haemolytica* with the reactions visualized on an 1.5% agarose gel
[8] *B. trehalosi=Bibersteinia trehalosi*
[9] *M. haemolytica=Mannheimia haemolytica*
[10] Threshold cycle score at the automated threshold
Sample testing positive for *Bibersteinia trehalosi*

DISCUSSION

This is the first known study directed at prevalence data for *Bibersteinia trehalosi* (*B. trehalosi*) housed in the nasopharynx portion of the upper respiratory tract of cattle in the United States. Additionally, the development of a *B. trehalosi* selective assay for qPCR has not yet been done. *B. trehalosi*, formerly known as *Pasteurella trehalosi*, is originally from the complex of species that was known as the *Pasteurella haemolytica* complex (Blackall et al., 2007). As noted in previous studies, *B. trehalosi* is a common respiratory pathogen of sheep, specifically bighorn sheep (Dassanayake et al., 2013, Fernández et al., 2016, Kugadas et al., 2016, Miller et al., 2013, Walsh et al., 2016). The possibility of detecting the organism before it gets to the lungs of cattle would be beneficial in the prevention of an infection.

Mannheimia haemolytica (*M. haemolytica*) and *B. trehalosi* are closely related, as *M. haemolytica* was also originally part of the *Pasteurella haemolytica* complex prior to reclassification through phylogenetic studies (Blackall et al., 2007, Klima et al., 2017). The relation between the two organisms was also confirmed in a study done in 2011 on the *tbpBA* operon of the two organisms. Through the research conducted, evidence of a common gene pool was made by showing various regions of the operon being shared by both *M. haemolytica* and *B. trehalosi* (Lee et al., 2011). This genetic association of the two organisms is where the current standards of detection stem from. Dassanayake et al. (2009) developed the multiplex assay used to differentiate between *B. trehalosi* and *M. haemolytica*. The *O*-sialoglycoprotein endopeptidase (*gcp*) and manganese-dependent superoxide dismutase (*sodA*) portions of the two organisms' sequences were aligned. This allowed for the production of species specific primer sets, using the *gcp* portion for *M. haemolytica* and the *sodA* portion for *B. trehalosi*. After PCR, the products of the primers were viewed via gel

electrophoresis where the bands would determine if a sample were *M. haemolytica* or *B. trehalosi*. These portions of the genomes are where the potential qPCR assay design of the current study began. Due to lack of specificity for *B. trehalosi* alone, the region used for the primer set was found elsewhere.

One attribute that distinguishes *B. trehalosi* from *M. haemolytica* is its ability to ferment trehalose (Blackall et al., 2007). While the exact gene that codes for this process was not known, the idea was to find the sequence that allows for this process to occur, in order to try and separate the two microorganisms. With that thought in mind the PTS trehalose transporter subunit IIBC was selected to use as the source of the primer sequences for the current study.

The ability of the assay to only detect amplifiable DNA from the first log dilution during the sensitivity trials; indicates that the assay can detect the presence of the bacteria when there are approximately six logs present in the initial extraction process. In order to improve this attribute of the assay, additional extraction methods could be explored such as; the QIAamp DNA minikit (Qiagen), Genomic-tip 100/G columns and buffers (Qiagen), and the use of InstaGene Matrix (Bio-Rad) with the harvest of one milliliter of cells by centrifugation, all of which were used in previous studies involving *B. trehalosi* (Anton et al., 2016, Kugadas et al., 2016, Lee et al., 2012). Additional ideas for improvement include an enrichment of the initial swab sample taken, or placing the swab on a Trypticase™ Soy Agar with 5% Sheep Blood (TSA II) plate. Isolation of a potential colony would then be required and the bacteria grown up in a broth, similar to what was done with the retest culture samples in this study. That would however prolong the identification process by adding additional steps.

Threshold cycle scores being reported for samples that were not found to be *B. trehalosi* indicates that the primer set is not specific to *Bibersteinia* alone. Further research should be conducted looking into possible DNA sequences that are unique to *B. trehalosi*. Klima et al. (2017) explored creating a multiplex PCR primer for *Mannheimia* species associated with BRD. In that study the gene targets were associated with the capsular polysaccharide or the lipopolysaccharide biosynthesis in order to distinguish between serotypes. This proved to be able to correctly identify three different serotypes of *M. haemolytica*; it is possible that the same area of the *B. trehalosi* genome can lead to a more specific assay. Likewise, Guenther et al. (2008) developed a qPCR assay specific to *Mannheimia* species by using the *sodA* gene previously mentioned. Though this gene was originally targeted for the primer sequence in this study, more intensive research could be done as it has proven to be successful for other specific assays (Guenther et al., 2008).

Since only one *B. trehalosi* was found out of the 188 samples taken utilizing PCR or traditional culture methods, it is unlikely that the microorganism is prevalent in the nasopharynx of cattle. Dassanayake et al. (2009) suggests that *B. trehalosi* can outgrow and even inhibit the growth of *M. haemolytica* in the lungs of bighorn sheep. This combined with *B. trehalosi* being an opportunistic bacterium could potentially explain why there is an increase in the appearance of the organism in the lungs of BRDC infected cattle. Additionally, it has been hypothesized that an increase in pathogenicity of *Bibersteinia* was acquired from other bacteria and is related to the genetic similarities. For instance, *B. trehalosi* and *M. haemolytica* share the pathogenic characteristic of the *lktA* gene, which is important in the expression of leukotoxin, a major virulence factor (Bowersock et al., 2014, Hanthorn et al., 2014). However, in a clinical trial evaluating the pathogenicity of *B.*

trehalosi, the results indicated that the pathogen may not be of primary importance and is a more opportunistic bacterium. In this study there were 12 calves inoculated intranasally with *B. trehalosi* or a combination of *B. trehalosi* and *M. haemolytica.* Of the 12 cattle, only one lung had *B. trehalosi* isolated from the tissue indicating that the bacteria isolates used in the study were not associated with significant disease in that research setting. Prior to the study, nasopharyngeal swabs of the calves did not find *Bibersteinia* to be present (Hanthorn et al., 2014).

In a study conducted on live export cattle in Australia, the prevalence of viral and bacterial agents associated with BRDC in nasal shedding was evaluated. In that study 1,484 nasal swabs were taken and examined for BHV, BRSV, PI-3, BVDV 1, Bovine Corona Virus, *Histophilus somni* (*H. somni*), *Pasteurella multocida* (*P. multocida*), *M. haemolytica* and *Mycoplasma bovis*. Though *B. trehalosi* was not tested for, the results of the study help in comparison of the other organisms examined for in the current study. The result for percentage of *M. haemolytica* presence was comparable to the current study at 13%, which was in the range of previously reported prevalence data (2-33%). *Pastuerella multocida* presence was higher compared to the current study as was *H. somni* (Moore et al. 2015). On the other hand, *Mycoplasma bovis* presence was greater in the current study when compared and was higher than the range of 0% to 43% previously reported by Moore et al. (2015). In a research review from 2009, it was reported that healthy feedlot cattle shed *Mycoplasma bovis* from the nasal passages, and approximately 50% of cattle have *Mycoplasma* present upon entering the feedlot; similar results were found with this study. Additionally, it has been reported that cattle that have been previously treated for BRDC have the highest percentage of *Mycoplasma bovis* pneumonia cases (Confer, 2009). However, in this study the New Pull

samples had a greater presence of *Mycoplasma* positive samples when comparing to the percentage of the Repeat samples.

Holman et al. (2015) conducted a study in Canada with the objective of studying the total microbiota in the nasopharynx of feedlot cattle using the 16s rRNA gene. Again, *B. trehalosi* was not looked for in this study, although *M. haemolytica, P. multocida,* and *H. somni* were. It was found that 10.6% of all the bacterial isolates on non-selective media were *Mannheimia* species (Holman et al. 2015).

In conclusion, the prevalence of *B. trehalosi* in the nasopharynx of feedlot cattle is low, suggesting that it is not a primary pathogen in cattle. At the current rate of 0.54% prevalence as detected by this study, there is not a great need for a specific qPCR assay. The further development of the assay may be beneficial for the sheep industry as the organism is considered a primary pathogen, and is found at a higher prevalence. If *B. trehalosi* prevalence increases in cattle, the optimization of a specific and sensitive assay could be of use.

LITERATURE CITED

Anton, B. P., G. P. Harhay, T. P. L. Smith, J. Blom, and R. J. Roberts. 2016. Comparative methylome analysis of the occasional ruminant respiratory pathogen *Bibersteinia trehalosi*. PLoS ONE. 11:e0161499. Available from: http://dx.doi.org/10.1371/journal.pone.0161499

Besser, T. E., M. Highland, K. Baker, E. F. Cassirer, N. J. Anderson, J. M. Ramsey, and J. A. Jenks. 2012. Causes of pneumonia epizootics among bighorn sheep, western united states, 2008–2010. Emerging Infect. Dis. 18(3):406–414. doi:10.3201/eid1803.111554

British Veterinary Association. *Bibersteinia trehalosi* infection in cattle and sheep. 2015. Vet Rec Case Rep. 176(8):195–198. Available from: http://dx.doi.org/10.1136/vr.h603

Blackall, P. J., A. M. Bojesen, H. Christensen, and M. Bisgaard. 2007. Reclassification of *Pasteurella trehalosi* as *Bibersteinia trehalosi*. Int. J. Syst. Evol. Microbiol. 57:666–674. Available from: http://dx.doi.org/10.1099/ijs.0.64521-0

Bowersock, T. L., B. E. Sobecki, S. J. Terrill, N. C. Martinon, T. R. Meinert, and R. D. Leyh. 2014. Efficacy of a multivalent modified-live virus vaccine containing a *Mannheimia haemolytica* toxoid in calves challenge exposed with *Bibersteinia trehalosi*. Am. J. Vet. Res. 75:770–776. Available from: http://dx.doi.org/10.2460/ajvr.75.8.770

Confer, A. W. 2009. Update on bacterial pathogenesis in BRD. Anim Health Res Rev. 10:145–148. Available from: http://dx.doi.org/10.1017/S1466252309990193

Dassanayake, R. P., D. R. Call, A. A. Sawant, N. C. Casavant, G. C. Weiser, D. P. Knowles, and S. Srikumaran. 2009. *Bibersteinia trehalosi* inhibits the growth of *Mannheimia haemolytica* by a proximity-dependent mechanism. J Appl Environ Microbiol. 76:1008–1013. Available from: http://dx.doi.org/10.1128/AEM.02086-09

Dassanayake, R. P., S. Shanthalingam, R. Subramaniam, C. N. Herndon, J. Bavananthasivam, G. J. Haldorson, W. J. Foreyt, J. F. Evermann, L. M. Herrmann-Hoesing, D. P. Knowles, and S. Srikumaran. 2013. Role of *Bibersteinia trehalosi*, respiratory syncytial virus, and parainfluenza-3 virus in bighorn sheep pneumonia. Vet. Microbiol. 162:166–172. Available from: http://dx.doi.org/10.1016/j.vetmic.2012.08.029

Drew, M. L., K. M. Rudolph, A. C. S. Ward, and G. C. Weiser. 2014. Health status and microbial (pasteurellaceae) flora of free-ranging bighorn sheep following contact with domestic ruminants. Wildlife Society Bulletin. 38:332-340. doi:10.1002/wsb.393

Fernández, S., J. Galapero, L. Gómez, C. J. Pérez, D. Cid, M. C. Martín, J. M. Alonso, and J. Rey. 2016. *Mannheimia haemolytica* and *Bibersteinia trehalosi* serotypes isolated from merino breed lambs in Extremadura (southwestern spain). Indian J Microbiol. 56(4):513-515. doi:10.1007/s12088-016-06117

Guenther, S., P. Schierack, M. Grobbel, A. Lübke-Becker, L. H. Wieler, and C. Ewers. 2008. Real-time PCR assay for the detection of species of the genus Mannheimia. Journal of Microbiological Methods 75:75–80. Available from: http://dx.doi.org/10.1016/j.mimet.2008.05.008

Hanthorn C. J., R. D. Dewell, V. L. Cooper, T. S. Frana, P. J. Plummer, C. Wong, and G. A. Dewell. 2014. Randomized clinical trial to evaluate the pathogenicity of *Bibersteinia trehalosi* in respiratory disease among calves. BMC Veterinary Research. 10:89. doi:10.1186/1746-6148-10-89

Harhay G. P., D. S. McVey, S. Koren, A. M. Phillippy, J. Bono, D. M. Harhay, M. L. Clawson, M. P. Heaton, C. G. Chitko-McKown, J. Korlach, and T. P. L. Smith. 2014. Complete closed genome sequences of three *Bibersteinia trehalosi* nasopharyngeal isolates from cattle with shipping fever. Genome Announc. 2(1):e00084-14. doi:10.1128/genomeA.00084-14

Holman, D. B., T. A. McAllister, E. Topp, A.-D. G. Wright, and T. W. Alexander. 2015. The nasopharyngeal microbiota of feedlot cattle that develop bovine respiratory disease. Vet. Microbiol. 180:90–95. Available from: http://dx.doi.org/10.1016/j.vetmic.2015.07.031

Kugadas, A., J. L. Humann, S. A. Pierlé, S. Srikumaran, and K. A. Brayton. 2016. Genome sequence of *Bibersteinia trehalosi* strain Y31 isolated from the pneumonic lung of a bighorn sheep. Genome Announc. 4:e00722–16. Available from: http://dx.doi.org/10.1128/genomeA.00722-16

Lee, I., and R. L. Davies. 2011. Evidence for a common gene pool and frequent recombinational exchange of the *tbpBA* operon in *Mannheimia haemolytica*, *Mannheimia glucosida* and *Bibersteinia trehalosi*. Microbiology. 157:123-135. Available from: http://dx.doi.org/10.1099/mic.0.041236-0

Miller M. W., B. M. Hause, H. J. Killion, K. A. Fox, W. H. Edwards, and L. L. Wolfe. 2013. Phylogenetic and epidemiologic relationships among *Pasteurellaceae* from colorado bighorn sheep herds. Journal of Wildlife Diseases. 49(3):653-660. doi:10.7589/2012-11-274

Moore, S. J., M. A. O'Dea, N. Perkins, and A. J. O'Hara. 2014. Estimation of nasal shedding and seroprevalence of organisms known to be associated with bovine respiratory disease in australian live export cattle. J. Vet. Diagn. Invest. 27:6–17. Available from: http://dx.doi.org/10.1177/1040638714559741

Newport, A. 2017. *Bibersteinia trehalosi* on the rise in beef cattle. (BEEF Vet). http://www.beefmagazine.com/animal-health/bibersteinia-trehalosi-rise-beef-cattle (Accessed 28 December 2017.)

Sacco, R. E., J. L. McGill, A. E. Pillatzki, M. V. Palmer, and M. R. Ackermann. 2013. Respiratory syncytial virus infection in cattle. Vet. Pathol. 51:427–436. Available from: http://dx.doi.org/10.1177/0300985813501341

Spagnoli, S., T. J. Reilly, M. J. Calcutt, W. H. Fales, and D. Y. Kim. 2011. Subcutaneous botryomycosis due to *Bibersteinia trehalosi* in a texas longhorn steer. Vet. Pathol. 49:775–778. Available from: http://dx.doi.org/10.1177/0300985811429309

Villard, L., D. Gauthier, F. Maurin, E. Borges, Y. Richard, G. Abadie, and A. Kodjo. 2008. Serotypes A1 and A2 of *Mannheimia haemolytica* are susceptible to genotypic, capsular and phenotypic variations in contrast to T3 and T4 serotypes of *Bibersteinia* (*Pasteurella*) *trehalosi*. FEMS Microbiol. Lett. 280:42–49. Available from: http://dx.doi.org/10.1111/j.1574-6968.2007.01035.x

Walsh, D., E. Cassirer, M. Bonds, D. Brown, W. Edwards, G. Weiser, M. Drew, R. Briggs, K. Fox, M. Miller, S. Shanthalingam, S. Srikumaran, and T. Besser. 2016. Concordance in diagnostic testing for respiratory pathogens of bighorn sheep. Wildlife Society Bulletin. 40:634-642. doi:10.1002/wsb.721

APPENDIX I

Recipe for Tryptic Soy Tryptose Broth (TSTB) (500ml) for Seed Tubes:

Add the following to a sterile Erlenmeyer flask with a stir bar:

15g of Tryptic Soy Broth

2.5g of tryptose

500ml of sterile RO water

Stir thoroughly on a stir plate

Aliquot 15ml into test tubes

Sterilize for 20 minutes at $\geq$ 121°C in an autoclave.

Remove from autoclave and allow to cool before use.

APPENDIX II

Recipe for Mycoplasma agar (1 liter):

Add the following to a sterile 2L Erlenmeyer flask with stir bar:

25g heart infusion agar

10g heart infusion broth

10g PP#3

0.25g thallium acetate

900ml sterile RO water

Mix thoroughly with heated stir plate.

Adjust pH of agar to 7.9 using 5M NaOH and 5M HCl.

Autoclave at $\geq$ 121°C for 20 minutes with slow exhaust.

After autoclaving, place in water bath preheated to 56°C for 30 minutes.

Allow to cool before adding the following:

126ml heat inactivated equine sera

100ml sterile yeast extract

21ml DPN-cysteine

0.635g Penicillin G (do not add when broth is hot as penicillin will curdle)

Mix thoroughly until Penicillin G dissolves.

Aliquot into petri dishes. (~7ml per small petri dish, ~55ml per large petri dish)

Allow plates to cool overnight before placing into refrigerator.